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MORPHOLOGY OF POLYMERIZED MEMBRANES ON AN AMORPHOUS SUBSTRATE AT MOLECULAR RESOLUTION BY AFM

by

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The morphology of lipid membranes on solid supports was studied by micro fluorescence filmbalance technique and Atomic Force Microscopy. The potential of AFM to visualize defects in lipid bilayers was demonstrated. The structure of polymerized membranes of the two lipids 10, 12-pentacosadiynoic acid (PC) and Dimethyl bis(pentacosadiynoyloxyethyl) ammonium bromide (BRONCO) was investigated with AFM. In both cases molecular resolution was achieved using amorphous silicon oxide as substrate. PC showed a highly regular packing of the hydrocarbon chains whereas in the case of the BRONCO film, which was polymerized in the high temperature phase, the positional correlation was only local.

Morphology of polymerized membranes on an amorphous substrate at molecular resolution by AFM

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Abstract

The morphology of lipid membranes on solid supports was studied by micro fluorescence filmbalance technique and Atomic Force Microscopy. The potential of AFM to visualize defects in lipid bilayers was demonstrated. The structure of polymerized membranes of the two lipids 10,12-pentacosadiynoic acid (PC) and Dimethyl bis(pentacosadiynoyloxyethyl) ammonium bromide (BRONCO) was investigated with AFM. In both cases molecular resolution was achieved using amorphous silicon oxide as substrate. PC showed a highly regular packing of the hydrocarbon chains whereas in the case of the BRONCO film, which was polymerized in the high temperature phase, the positional correlation was only local.

Introduction

Supported planar lipid-protein-membranes have been shown to be relevant model systems for cell surfaces,[1] which due to their complexity and flexibility can hardly be accessed directly by surface sensitive techniques. With a suitable choice of the components, however, the lateral as well as the transverse mobility of the molecules in or at a supported membrane can be drastically reduced[2]. This allows the investigation with near-field techniques giving rise to molecular resolution[3]. The design of such a supported planar membrane can be in a way that it resembles a natural membrane to such an extent that it is accepted by other cells as communication partner[4].

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Conversly, such supported planar membranes have several essential features making them ideally suited substrates for the immobilization of certain molecules to be imaged by scanning probe techniques like AFM[5]. First, on suitable supports these membranes are flat on a macromolecular scale (some hundred Å) and second, they can be designed in a way that they are biologically neutral surfaces not unfolding or denaturing proteins[6]. Third there exists an established technology which allows the control on the two-dimensional thermodynamics of the films[7] and fourth, there is a broad variety of natural as well as synthetic lipids available. For the purpose of the immobilization of large molecules two strategies are conceivable: the design of charge pattern[8] as well as the use of reactive lipid headgroups for chemical binding[6]. In both cases the stability of the membranes is a crucial parameter. The stability of such membranes can be drastically increased by the use of polymerizable lipids which form solid two-dimensional networks. Such polymerized membranes can be imaged with the AFM, giving rise to molecular resolution even on amorphous substrates.

Materials and Methods

Lipids: 10,12-pentacosadiynoic acid (PC) was purchased from Farchan Laboratories, Karlsruhe FRG and was recrystallized several times in 5:1 hexane / ethanol (Merck Darmstadt). Dimethyl bis(pentacosadiynoyloxy-ethyl) ammonium bromide (BRONCO) was synthesized from PC following the procedure given in Ref. 9. The polymerizable lipids were freeze dried and stored in the dark. Dimyristoylphosphatidylglycerol (DMPG) and arachidic acid were purchased from Sigma and used without further purification. For the Langmuir-Blodgett experiments, all lipids were dissolved in chloroform (HPLC grade, Aldrich) to a final concentration of about 1 mMol.

Supports: Ruby mica sheets were freshly cleaved before they were used. The silicon wafers were a kind gift from Wacker Burghausen. According to Wacker, these wafers are especially processed resulting in an extraordinarily flat surface of the oxide layer which has a thickness of about 2000Å. We have checked several samples by AFM and confirmed that the surface roughness is less than 1Å on a 100Å scale as judged by the use of integrated tips.

Monolayer experiments: A micro fluorescence Langmuir-Blodgett apparatus built in our laboratory was used for the recording of the pressure area diagrams as well as for the film deposition[10]. As subphase either pure water (millipore quality), HEPES (10mM HEPES + 10mM NaCl, pH 8.3), or a $5x10^{-4}$ molar $CdCl_2$ solution pH 7.5 was used. The lipids were applied to the air water interface from chloroform solution, allowing the solvent to evaporate for several minutes prior to compression. Both mica and silicon wafer were coated with a Cd-arachidate monolayer by vertical deposition (100 µm/sec) at a lateral pressure of π = 30 mN/m. The upper monolayer was deposited by horizontal dipping. The polymerization of the films was

performed by UV irradiation for about one minute (Hg pen ray) and was assumed to be completed when the lateral pressure stopped dropping.

Atomic Force Microscopy: A detailled description of the AFM is given elsewhere[11]. It was driven by a Macintosh IIci equipped with a GW Instr. Inc. AD/DA board. Data acquisition was accomplished by specially developed software. Image analysis was done with an extended version of Image from W. Rasband, NIH, Bethesda. Cantilevers with integrated tips were purchased from Park Scientific Instruments, Mountain View, California.

Results and Discussion

Supported lipid membranes have previously been imaged by STM[12] as well as AFM[8,13]. We have shown that under certain conditions molecular resolution can be achieved. Although two dimensional lipid crystals have proved to be useful candidates for the immobilization of larger molecules like DNA or proteins, their lack of stabiiity in both time, mechanical durability limits the range of possible applications. As an example, in Fig 1a shows holes in a supported membrane.

Fig. 1
AFM image of holes in an asymmetric supported planar membrane taken at room temperature in HEPES buffer. The upper leaflet of the bilayer consists of the negatively charged DMPG. The lower leaflet consists of a Cd-arachidate layer. Mica was used as the substrate.

Such holes can be caused by defects in the lower half of the bilayer consisting of Cd-arachidate. In a previous paper, we have shown that defects in the first layer can occur during the transfer process or upon aging[14]. As long as these defects are small enough, the upper monolayer can stretch over them. If the extension of these defects is too large or if the mechanical stress during imaging is too high, these defects destabilize the upper monolayer resulting in a local breakdown of the membrane. The lateral surface tension in the membrane enlarges the holes spanning both monolayers. The resulting perimeter of the hole is determined by an equilibrium between surface and rim tension. As the rim is one-dimensional this equilibrium is extremely sensitive to trace amounts of impurities[15] such as lysolipids, which as breakdown products are always present to a certain extend in lipids. The formation of such holes in membranes can also be induced deliberately by locally scanning at high forces. This process might be of some benefit for certain applications in the design of nano structures.

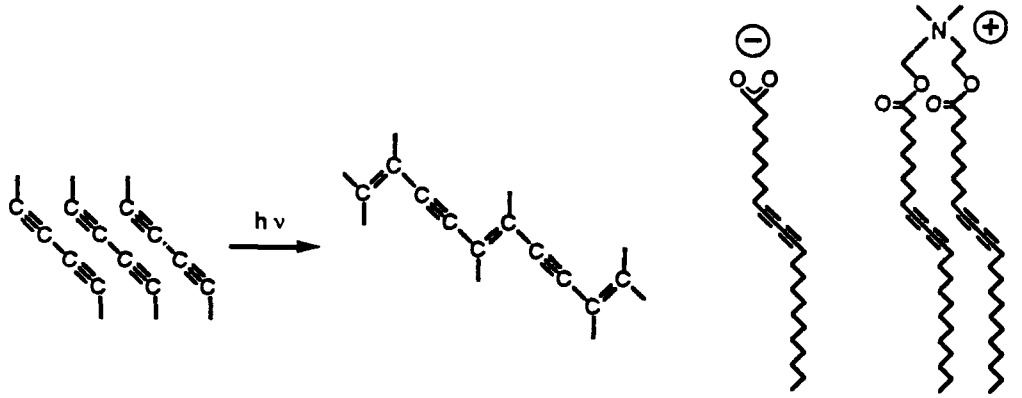


Fig. 2
a) Schematics of the topochemical p[illegible]merization b) PC c) BRONCO

In order to overcome these problems we investigated the use of membranes stabilized by in-plane chemical crosslinking of the lipids thus preventing a local reorganization of the films. There is a large body of literature mainly from the late seventies on these polymerizable lipids. Several investigators have studied all types of reactive groups and strategies for the formation of such linear, branched, or network-like two-dimensional polymers[16]. Among all polymerizable lipids the ones carrying diynoic groups in the hydrocarbon chains have been most well-characterized. The major reason for this is that these groups after polymerization form strong dyes,[17] which exhibit an intrinsic fluorescence with transition dipole moments pa[illegible]el to the poly[illegible]r backbone. The underlying so-called topochemical pc[illegible]erization reactic[illegible]s depicted in Fig. 2a. For this type of polymerization to [illegible]ccur, the reactive groups have to be aligned in a certain relative orientation. In the case of the polymerizable lipid BRONCO and the fatty acid PC (Fig. 2b,c) this restriction is fulfilled when the chains are packed in a two-dimensional crystal.

One way to achieve this packing is to crystallize the polymerizable lipids at the air-water interface of a Langmuir-Blodgett trough under suitable conditions of temperature, ionic strength, and pH of the subphase. The resulting pressure area diagrams are shown in Fig. 3a. The pressure-area diagram of BRONCO shows a horizontal deflection at $\pi \approx 16$ mN/m indicating a fluid/solid coexistence. The film was polymerized by UV irradiation at the pressure indicated by the arrow. The resulting micro fluorescence image taken from the air-water interface (Fig. 3c) shows bright crystalline domains, which do not redissolve upon decompression indicating that indeed two-dimensional crystalline polymers have formed. The macroscopic morphology of these crystalline domains reflects certain features of the microscopic arrangement of the lipids, but this correlation is not yet fully understood[18]. In the case of the polymerizable fatty acid the fluid/solid coexistence occurs at a somewhat lower pressure ($\pi \approx 9$ mN/m). The formation of elongated crystals can again be visualized with the fluorescence microscope after polymerization (Fig. 3b).

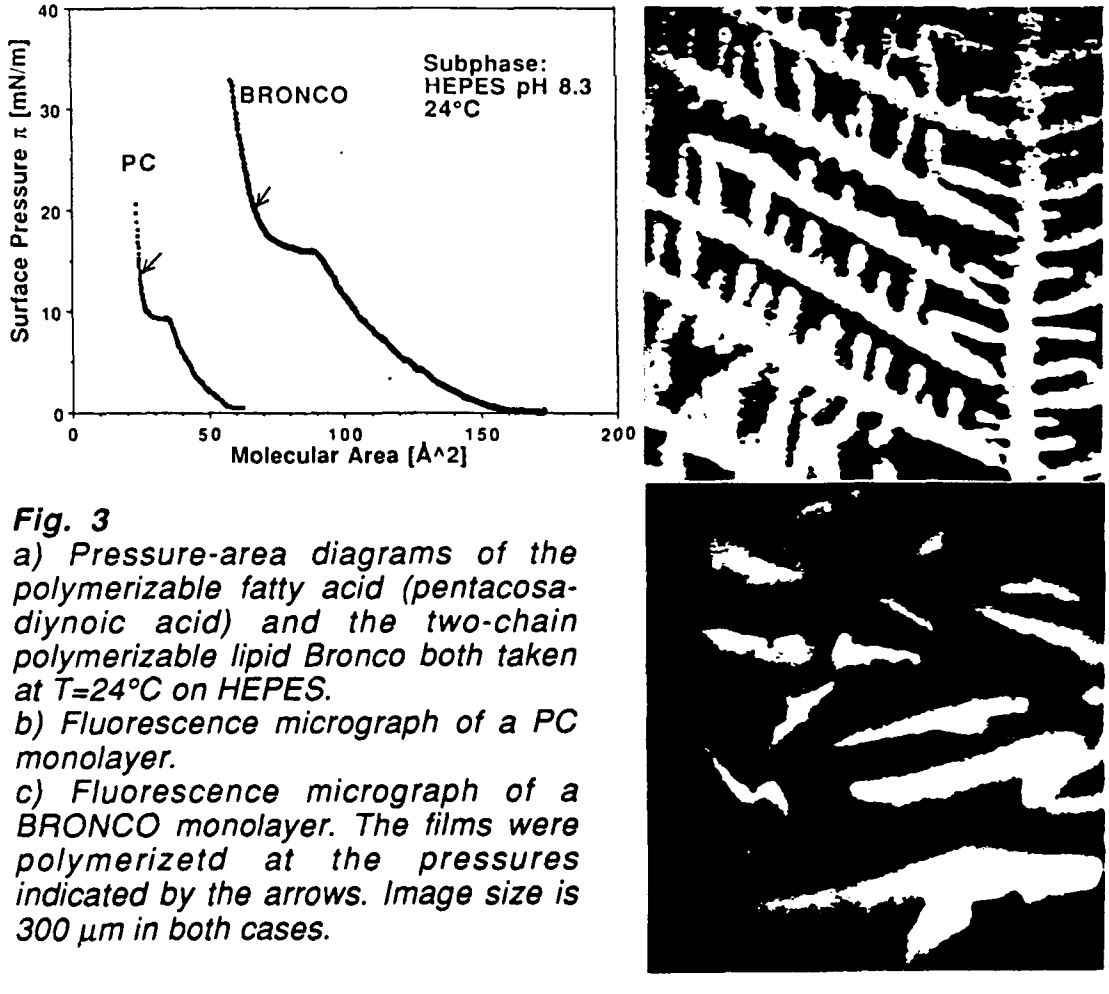


Fig. 3

a) Pressure-area diagrams of the polymerizable fatty acid (pentacosa-diynoic acid) and the two-chain polymerizable lipid Bronco both taken at T=24°C on HEPES.

b) Fluorescence micrograph of a PC monolayer.

c) Fluorescence micrograph of a BRONCO monolayer. The films were polymerizetd at the pressures indicated by the arrows. Image size is 300 μm in both cases.

These films were transferred onto silicon wafers which had been precoated with a monolayer of Cd-arachidate. This monolayer is known to form stable and tightly packed crystalline films bound to the negatively charged surface via a Cd salt bridge. These wafers have a thermally grown oxide layer, which due to a special processing is extremely flat. The resulting bilayers were kept submerged and were imaged with the AFM.

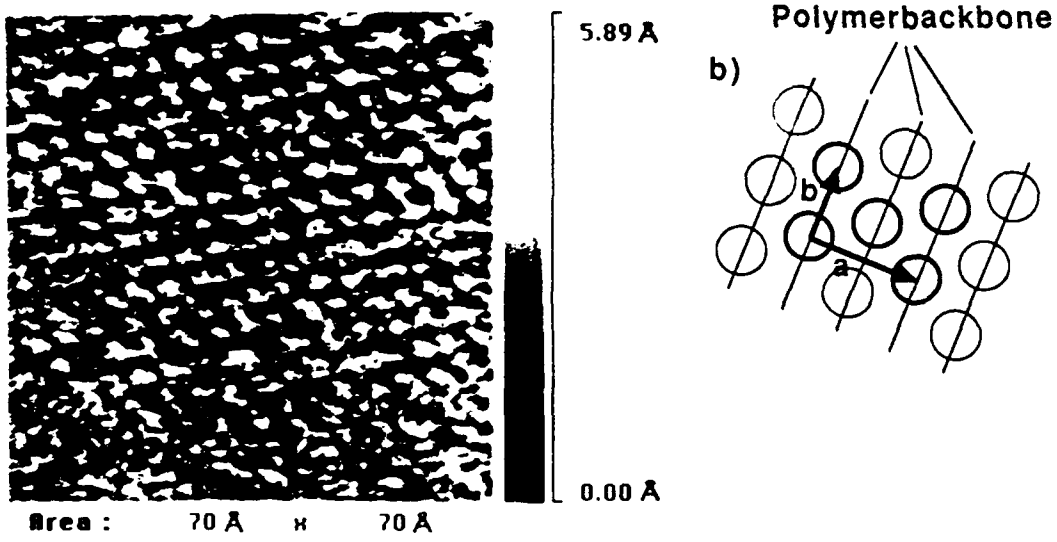
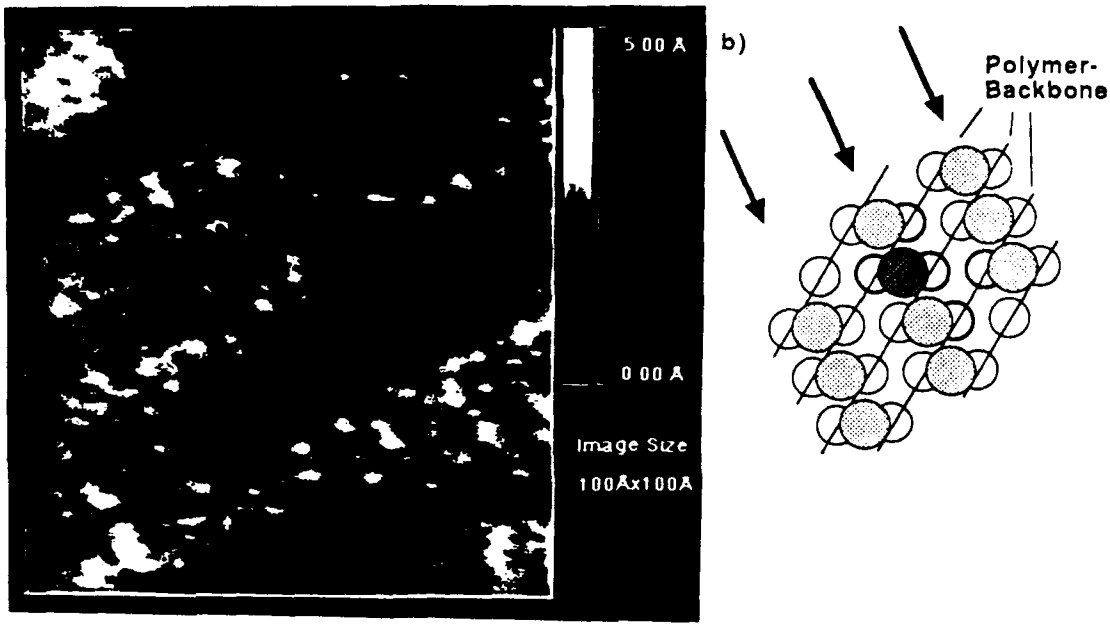


Fig. 4
a) Molecular resolution AFM image of an asymmetric supported membrane of the polymerized fatty acid on Cd-arachidate. In this case a silicon wafer with thermally grown oxide was used as the substrate. The image was taken in HEPES buffer at room temperature.
b) Molecular model for the image above.

The AFM image for the polymerized PC film is given in Fig. 4. It shows clearly distinguishable bumps of about one Å in height which are arranged in a slightly distorted hexagonal lattice. From electron diffraction and x-ray reflectivity measurements on similar monolayers in air or vacuum, it is known that such a distortion may stem from a slight tilt of the hydrocarbon chains[21]. In measurements on multilayers an orthorhombic chain lattice with a = 7.4 Å, b = 4.9 Å was found[19]. Within experimental error (≈10%), this is also the lattice that we measure with AFM in aqueous environment. These data are also in good agreement with the lattice that we have previously found for the same molecule but with mica as the support[13]. These results show unambiguously that such highly ordered organic films can be imaged with at least molecular resolution. Earlier experiences with quartz as the support[20] have lead us to the conclusion that the surface roughness of the support may play a crucial role for the resolution at which such films can be imaged. This is now confirmed by these measurements where we found molecular resolution on an amorphous but extremely flat support.



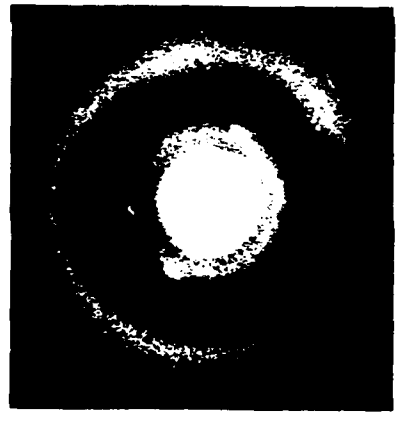


Fig. 5
a) AFM image of a BRONCO monolayer polymerized in the high- temperature phase and transferred onto Cd-arachidate on a silicon wafer with thermally grown oxide. The image was again taken in HEPES buffer at room temperature.
b) Molecular model for the image
c) Electron diffraction pattern of a polymerized monolayer consisting of lipids very similar to BRONCO. This image was taken from Göbel et al. (Ref 21).

Another example for the potential of AFM to resolve structural details of such films at the molecular level is shown in Fig. 5a. Here a film of polymerized BRONCO was imaged. Earlier micro fluorescence and electron diffraction studies on a very similar molecule have shown that the two-dimensional crystals from this class of molecules[21] exhibit a distinct difference in morphology and internal structure above and below a transition temperature of about 20°C. In the high-temperature phase, the packing of the hydrocarbon chains is less dense than in the low temperature phase and shows a coherence length of only some tens of molecules. After polymerization, the lattice experiences an additional source of distortion. The hydrocarbon chains have to arrange from an average area per chain of 28Å^2 which corresponds to half the molecular area of the fully compressed film in Fig. 2, to an area of 22Å^2 in the polymerized state as discussed above. Electron diffraction on such polymerized monolayers gives the pattern

shown in Fig. 5a. The most interesting feature here is that the reflexes are not spots but streaks. These streak-like reflexes were interpreted in a way that in the high-temperature phase the order of the lipids is well preserved in one direction presumably along the polymer backbone and less well pronounced in the other directions[21]. From the correlation between the macroscopic crystal morphology and the fluorescence polarization indicating the direction of the polymer backbone one can predict the orientation of the headgroup with respect to the polymer axis. The above interpretation can be summarized in the model shown in Fig. 5b.

We can directly compare this model with the image obtained by AFM. The AFM image shows that both predictions are correct. The position correlation of the lattice is only locally preserved and well expressed in just one direction. The spacing of the rows (indicated by the arrows in Fig. 5a) is about 7.5Å, which is the value one would expect for the distance between the rows of the headgroups in the direction parallel to the polymer backbone. On a large scale, the orientational correlation between the well-ordered areas is preserved, which is essential for the expression of a distinct morphology of the crystalline domains and the observed homogeneous fluorescence polarization.

Concluding Remarks

We have shown before that molecular resolution images of lipid films on mica can be achieved by AFM. Here we demonstrate that this is also possible on amorphous substrates provided they are flat enough. We have chosen two polymerizable lipids carrying opposite charge because such molecules may be laterally arranged, e.g., by cocrystallization to form certain charge pattern which then can be stabilized by polymerization. Such two-dimensional polyelectrolytes should by themselves be extremely interesting new materials with designable properties and they should also offer very useful applications, e.g., for the orientation and immobilization of macromolecules at surfaces.

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